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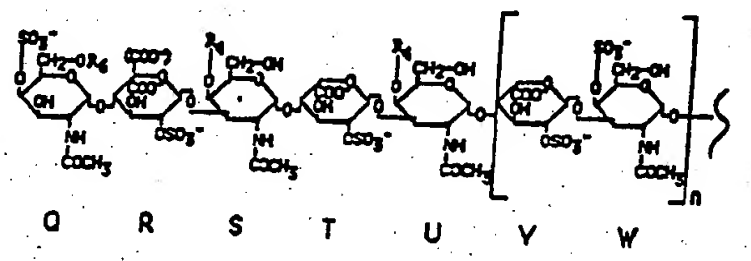
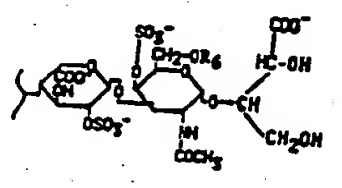
(54) Title: OLIGOSACCHARIDES OF DERMATAN SULPHATE, PROCESS FOR THEIR PRODUCTION, AND ASSOCIATED PHARMACEUTICAL COMPOSITIONS

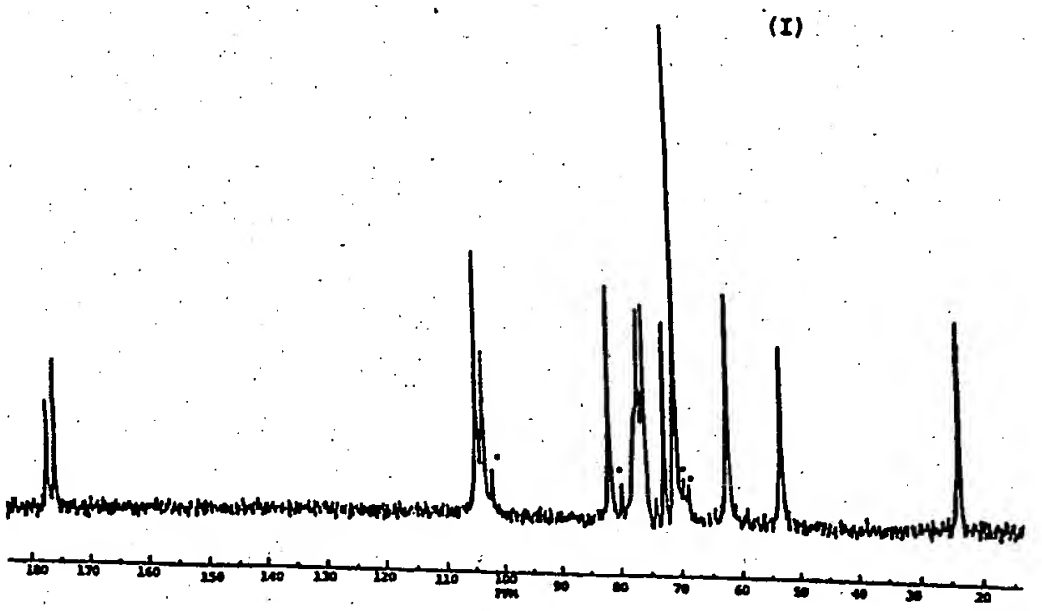


Q R S T U V W

(I)



X Y Z



(57) Abstract

Oligosaccharides of dermatan sulphate, of formula (I) (where n = 0, 1 or 2; Q, S, U, W and Y = residues of N-acetylgalactosamine 4-sulphate or 4,6-disulphate; R, T, V and X = residues of L-iduronic acid 2-sulphate with at least one possible variant of D-glucuronic acid 2-sulphate), have valuable antiarthritic, anti-inflammatory, antithrombotic and antiatherosclerotic properties.

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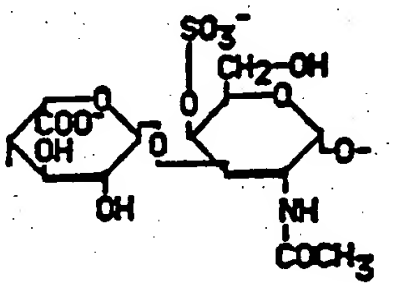
OLIGOSACCHARIDES OF DERMATAN SULPHATE, PROCESS FOR THEIR PRODUCTION, AND ASSOCIATED PHARMACEUTICAL COMPOSITIONS

The invention relates to oligosaccharides of dermatan sulphate having biological properties and more particularly having the property of inhibiting the movements of the Ca ion in human platelets incubated with polymorphonucleate autologous leukocytes stimulated by chemotactic agents. This particular action comes within the more general framework of a pharmacological activity such as to inhibit inflammation, platelet aggregation and certain contributory causal factors in thromboembolic diseases. The invention also concerns a process for obtaining the said products, and pharmaceutical compositions containing the said products as the active principle.

Dermatan sulphate has always been regarded as a heterogeneous polysaccharide consisting mainly of the disaccharide (II)

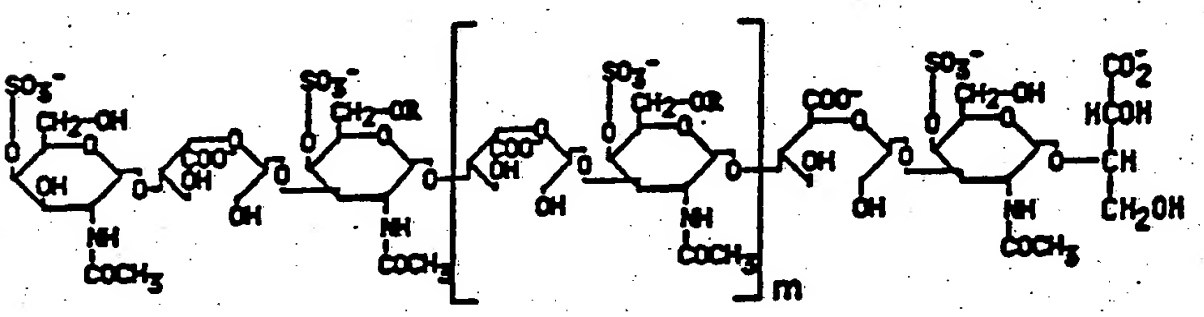


(II)

European Patent 97,625 claims a process for the preparation of dermatan sulphate from mixtures of polysaccharides, the said dermatan sulphate having an anticongestive and cicatrising action. Patents EP A 199,033 and EP A 238,994 claim a process for the extraction and purification of dermatan sulphate and

its use as an antithrombotic drug in the prophylaxis of venous thromboses.

EP 221,977 claims inter alia dermatan sulphate fractions having molecular weights lying between 2000 and 7000 Daltons, but more particularly between 4000 and 6000 Daltons, obtained by radical depolymerisation of dermatan sulphate. Likewise EP A 269,937 claims low molecular weight glycosamine-glycans obtained by gamma radiations. The products claimed in the above-mentioned patents are acclaimed as having a pharmacological and clinical activity in the prophylaxis of venous thromboses. Application PCT EP/89/01214 of the present applicant describes a process of oxidation and reduction of the C_2-C_3 bonds of the uronic acids of dermatan sulphate, in which the non-sulphated uronic acids (practically all of them) are oxidised to dialdehydes and then reduced to hydroxymethyl derivatives. The said patent then claims fractions of the general formula (III)



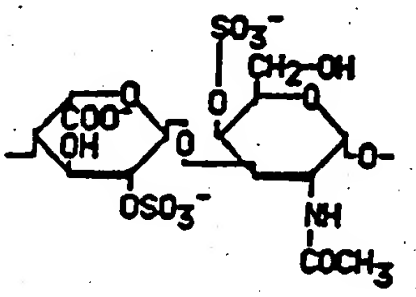
(III)

where m can vary so as to include fractions of molecular weight from 9400 to 22000 Daltons, and the specific rotation $[\alpha]^{20}_{D}$ lies between -13° and -5°.

The glycosamine-glycans located on the cell surfaces or in the extracellular matrices, and among these dermatan sulphate in particular, interact with heparin cofactor II (HCII) to inhibit the proteolytic

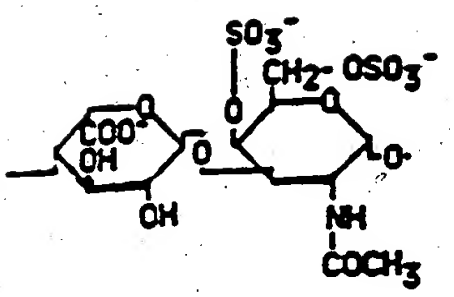
activity of the thrombin involved in blood coagulation: in this connection see D.M. Tollefsen in "The Metabolic Basis of Inherited Disease" (Scriver C.R., Beaudet A.L., Sly W.S. and Valle D. ed.s), 6th Ed., pp. 2207-2218, Mc Graw-Hill, New York (1989). A hexasaccharide, containing iduronic acid 2-sulphate, Ido-(2-SO_3^-), obtained by nitrous acid deamination of a partly N-deacetylated dermatan sulphate, has been reported to be the oligosaccharide sequence of dermatan having the highest affinity for heparin cofactor II (HCII) (M.M. Maimone, D.M. Tollefsen, J. Biol. Chem. 265 (30), 18263-18271 (1990)).

Surprisingly it has now been found that the particular sequence contained in dermatan sulphate and believed by Maimone and Tollefsen to coincide with the active site for interaction with HCII is much more complex than described, and that this highly sulphated sequence is contained in a cluster. Whereas it had hitherto been thought that the disulphated disaccharides (1->4)-O-(2-O-sulpho-α-L-idopyranosyluronic acid)-(1->3)-O-(2-acetamido-2-deoxy-ß-D galactopyranosyl 4-sulphate (IV)



(IV)

and (1->4)-O-(α-L-idopyranosyluronic acid)-(1->3)-O-(2-acetamido-2-deoxy-ß-D-galactopyrosanosyl 4,6-bis-O-sulphate (II')



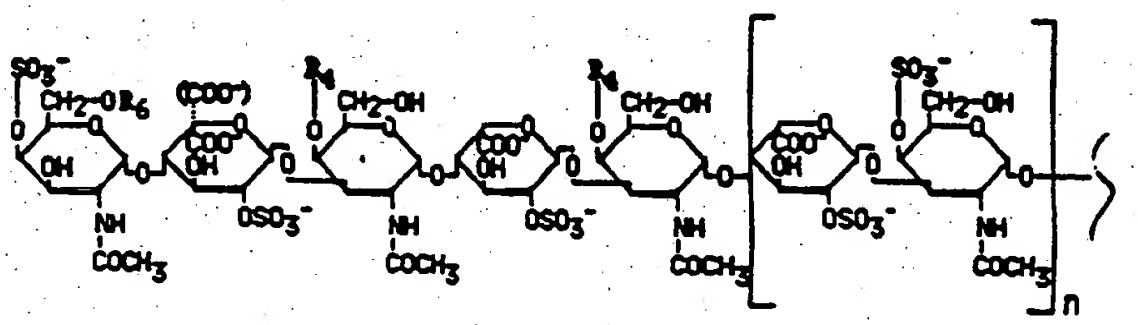
(II')

were contained at random and discontinuously in the structure of dermatan sulphate, it has surprisingly been found that the highly sulphated oligosaccharide sequences, obtained by a process that is also a subject of the invention, once isolated from the structural context of dermatan sulphate, no longer have any activity on heparin cofactor II, but exhibit therapeutically valuable pharmacological activities in the treatment of inflammatory, arthritic, thrombotic and atherosclerotic processes.

These compounds have in fact proved to be active in inhibiting production of free radicals by leukocytes, adhesion of polymorphonucleate leukocytes to the endothelium, platelet aggregation and cytoplasmatic movements of calcium in human platelets coincubated with PMNs stimulated by a chemotactic agent. These biological actions are of therapeutic value in diseases of chronic inflammatory (arthritic) or thrombotic type and in cardiovascular diseases in general. The pharmacological activity has proved to be considerable, being at least double or triple than that of other oligosaccharides in the heparin family, and unexpected since it was known, at least with regard to heparin, that the pentasaccharide of the active site of antithrombin III (ATIII) is of pharmacological importance solely in so far as it is inserted in a long

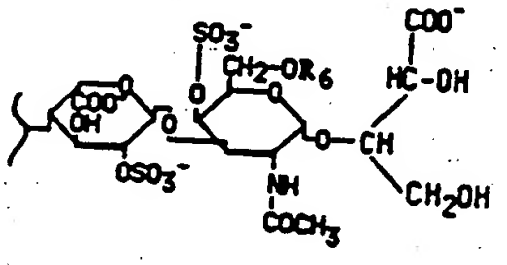
heparin polysaccharide chain and solely in so far as it possesses anticoagulant pharmacological activity.

One subject of the invention comprises novel oligosaccharides and fractions containing them. The oligosaccharides of the invention contain galactosamine N-acetylate-4-sulphate (and possibly also 6-sulphate) and L-iduronic acid 2-sulphate with at least one possible variant of a D-glucuronic acid 2-sulphate. The oligosaccharides of the invention have one unit of N-acetylgalactosamine 4-sulphate on the "non-reducing" side and a 2,3,4-trihydroxybutyric acid residue, the remnant of demolition of a uronic acid, indicated by Z, in the "reducing" termination. The oligosaccharides of dermatan sulphate that form the subject of the present invention have a structural formula as shown diagrammatically by sequence (I)



Q R S T U V W

(I)



X Y Z

in which:

n = 0, 1 or 2;

R_4 = SO_3^- or H;

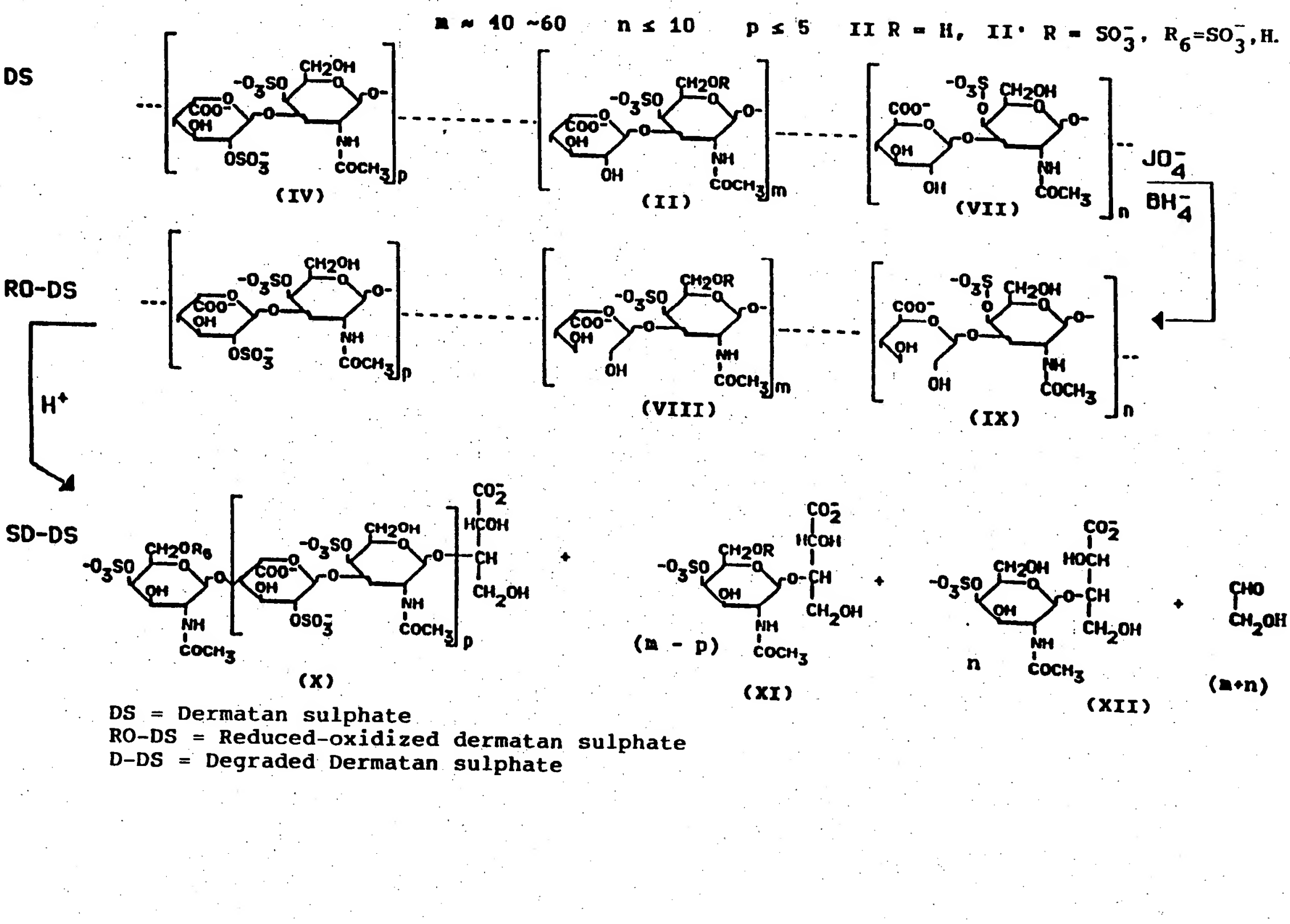
R_6 = H or SO_3^-;

Z = remnant of demolition of a uronic acid;

Q, S, U, W and Y = galactosamines;
R, T, V and X = residues of iduronic acid 2-sulphate, at least one of them however being possibly a residue of glucuronic acid 2-sulphate.

The oligosaccharides of the invention include the structures symbolised by Q, R, S, T, U, V, W, X, Y and Z. One oligosaccharide of the present invention is formed by the sequence QRSTUVWXYZ. Other oligosaccharides consist respectively of the sequences QRSTUXYZ, STUVWXYZ, and UVQXUZ, where Q and Y can be either N-acetylgalactosamine-4-sulphate or N-acetylgalactosamine-4,6-disulphate. The said oligosaccharide fractions of dermatan sulphate are of a type obtainable by a process including the following reactions, as indicated in Diagram 1 below:

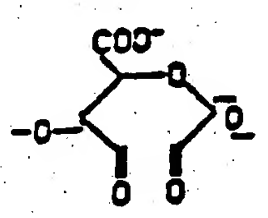
Scheme 1. Major disaccharide sequences of dermatan sulphate and products of acid degradation (arbitrary sequences).



DS = Dermatan sulphate
RO-DS = Reduced-oxidized dermatan sulphate
D-DS = Degraded Dermatan sulphate

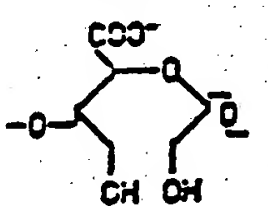
PCT/EP92/01791

- dermatan sulphate (DS) having the characteristics described in Italian Patent Application No. MI 91 A 002310, filed on the same date, and containing a high percentage of disulphate disaccarides (IV), measurable by enzymatic hydrolysis with chondroitinase ABC and evaluation of corresponding unsaturated disaccharide Δ Di-diS$_B$, and characterised by a 13C-NMR spectrum having the characteristic minor signals I^*_1 (δ 102), I^*_2 (δ 80), I^*_4 (δ 74), I^*_3 (δ 69.5), I^*_5 (δ 68.6), is subjected to oxidation with periodic acid in a neutral medium;
- the oxidation product obtained, having the C_2-C_3 bonds of the non-sulphated uronic acids oxidised to dialdehydes of type (V)



(V)

is reduced with sodium borohydride to a di(hydroxymethyl) derivative (VI)



(VI)

- the product obtained, designated "oxidised and reduced dermatan sulphate" (RO-DS), is subjected to controlled acid hydrolysis which gives rise only to hydrolysis of the semi-acetal bonds between uronic acids, with the C_2-C_3 bonds oxidised (open), and amino-sugars.

Not very severe conditions would give rise to medium-long chain fragments lacking uniformity; over-severe conditions would give rise to hydrolysis of all

the semi-acetal bonds between the sugars and certainly also to hydrolysis of the ester bonds of the sulphate groups. Other subjects of the present invention are therefore the conditions for acid hydrolysis conducted with acid (polysulphonic) resins so as to guarantee the acidity and ionic strength conditions suitable for hydrolysing the weaker semi-acetal bonds only and leaving the highly sulphated sequence contained in the dermatan sulphate unchanged.

EXAMPLE 1

a) Preparation of dermatan sulphate

Grind up 65 kg of bovine intestinal mucosa and place in reactor with 75 litres of water. Heat to 60°C and add 430 g of sodium chloride and 150 g of papain 1:350 (Merck). Correct pH to 6.3 with sodium acetate. Keep the mass stirring for 4 h at 60°C. After 3.5 hours there is already no further development of free amino acids in the proteolytic process. Heat the mass to 78°C for 10' and then filter through a filter-press on a filtering auxiliary (dicalite). 120 litres of filtrate are obtained. Percolate the solution at 45°C in a chromatographic column 10 x 80 cm containing Lewatite MP 5080 in Cl^- form. Remove the percolate. Wash the resin with 12 litres of 0.6 M NaCl solution, then elute with 18 litres of 1.8 M NaCl solution, with a flow rate of 2.5 BV/h (Bed Volume/h). Collect the solution and concentrate to 5 litres and to 1 M NaCl through reverse osmosis. Add 0.6 volumes of ethanol to the solution. Leave to stand for 10 hours at 5°C. Filter. To the filtrate add another 7 litres of ethanol. A precipitate of raw dermatan sulphate is

obtained; collect by filtering. Redissolve the solid in 1.5 litres of water brought to 0.5 M with copper acetate. To this solution add 100 g of dihydrated rameic chloride and 3 litres of ethanol. On standing, a precipitate is formed, which is collected by filtering, washed for a long time with ethanol, redissolved in 0.5 l of water acidified to pH 5.5 with acetic acid, and passed over a chromatographic column 4 x 25 cm containing Chelex 100 (BioRad) in H^+ form. The solution obtained from the column is salified with NaOH to pH 6.2 and lyophilised. 39.6 g of pure dermatan sulphate are obtained. The following analyses are performed on the dermatan sulphate:

- molecular weight (MW, by HPLC over Protein Pack 125-300 columns, mobile phase 0.125 M Na_2SO_4 and 2 mM NaH_2PO_4 at pH 6, on 3rd degree polynomial calibration curve obtained with molecular weight standards);
- sulphur percentage, uronic acids percentage, SO_3^-/COO^- (by potentiometric method according to Mascellani et al., Il Farmaco Ed. Pr. 43, 165 (1988));
- disaccharide composition according to Yoshida et al. (Anal. Biochem. 177, 327 (1989));
- 13C-NMR;
- in vitro activity on HCII evaluated by chromogenic method and with Stachrom D.S. kit (Diagnostica Stago containing CBS 34.47 substrate) according to Dupoury et al., Thromb. Haemost., 60, 2, 236 (1988)) against WHO's heparin 4th Standard at 193.4 IU/mg;
- in vivo antithrombotic activity evaluated in the vena cava ligature model in the rat, according to Rayers et al., Thromb. Res. 18, 699 (1980).

The DS obtained has the following characteristics: MWp 25,600 d (MWw = 28,500, MWn = 23,600, D = 1.21); sulphur 5.94; uronic acids 33%; SO_3^-/COO^- = 1.09; $[\alpha]^{20}_D$ = -60°; disaccharide composition: Δ DiOS = 0.9%; Δ Di6S = 2.7%; Δ Di4S = 84.7%; Δ Di-2,6diS = 0.3%; Δ Di-2,4diS = 9.2%; Δ Di-4,6diS = 2.0%; activity on heparin co-factor II a-HCII = 245 U/mg; ED50 = 1.02 mg/kg i.v. The DS has the 13C-NMR spectrum in Fig. 1.

b) Preparation of reduced-oxidised dermatan sulphate

Dissolve 100 g of dermatan sulphate, obtained according to a), in a liter of water, and slowly add 800 ml of a 0.5 M solution of $NaIO_4$. Leave the solution stirring for 4 hours, then cool in thermostatic bath at 10°C, bring to pH 8 with N NaOH and very slowly, with stirring, add 80 g of $NaBH_4$ in the period of 4 hours. At the same time add dilute acetic acid so that the pH remains in the range 7.5-8.5. Allow to stand for one night, bring the pH to 4 with 8 N HCl in order to favour the destruction of the excess $NaBH_4$ by stirring for an hour at room temperature. Then bring the pH to 5.5 with NaOH. Precipitate the product twice with three volumes of ethanol, equal to six liters per time. After filtering and drying 80 g of oxidised and reduced dermatan sulphate (RO.DS) are obtained and coded OP723. The RO.DS has the following characteristics: MW = 15,200; sulphur 5.9%; uronic acids 32.4%; SO_3^-/COO^- = 1.1; $[\alpha]^{20}_D$ = -8°.

c) Fractionation of RO-DS by molecular weight

5 g of the product designated OP723, obtained as above, were fractioned by gel permeation on

Ultrogel AcA54 (IBF) contained in a 5 x 84 cm column.

Chromatography was carried out in 1N NaCl with a rate of flow of 2 ml/minute. The effluents were monitored with UV detector equipped with continuous flow cell. 20 ml fractions were collected, and 7 pools of about 150-200 ml each were made. Collection of the fractions in pools was carried out on the basis of the absorbency values recorded on the detector.

The fractions were concentrated in a rotary evaporator to a volume between 80 and 120 ml. The precipitated sodium chloride was removed by filtering. The solutions, brought to pH 5.3 with acetic acid, were treated with two volumes of ethanol. The oily precipitate was redissolved in water and reprecipitated twice more with ethanol and acetone; when necessary the products obtained were desalted on Trisacryl CF 05 M (IBF) in a 5 x 38 cm column with a flow rate of 5 ml/minute. The solutions obtained were concentrated and the products again precipitated with ethanol. All fractions were found to be free from sodium chloride in the $AgNO_3$ test. The products coded LL 108 were obtained and are shown in Table I. The physicochemical characteristics of the various fractions are also given.

TABLE I

Fractions	1	2	3	4	5	6	7
g	0.35	1.0	2.1	0.2	0.90	0.1	0.14
% yield	7	20	42	4	18	2	2.8
MW(kd)	25.7	15.6	11	7.9	6.7	6	5.8
$[\alpha]20_D$			-8.5°	-8.6°	-8°		
SO_3^-/COO^-	1.04	1.08	1.11	1.08	1.05	1.03	1.03
a HCII (U/mg)	233.8	252.6	269.2	310.9	257	201.8	183.4

Notes - MW = molecular weight evaluated by HPLC on Protein Pak 300-125 columns (Waters), mobile phase 0.125M Na_2S04 and 2mM NaH_2PO_4 at pH 6 on 3rd order polynomial setting curve obtained with molecular weight standards.

- SO_3^-/COO^- obtained by potentiometric method (Il Farmaco Ed.Pr 43, 165 (1988)).

A-HCII activity on the second heparin cofactor in U/mg calculated by chromogenic method with the Stachrom kit (Diagnostica Stago) against the 4th WHO standard of heparin at 193.4 IU/mg. The 13C NMR spectrum of the LL 108/5 fraction is given in Fig. 2. Activities on heparin cofactor II are given in Fig. 3. This activity was determined according to the rapid colorimetric assay of dermatant sulphate; Stachrom D.S., on CBS 34.47 substrate (Dupouy D. et al., Thromb. Haemost., 60, 2, 236, 1988). Fraction 5 subjected to enzymatic attack with chondroitinase ABC (E.C.4.2.2.4) and subsequent HPLC evaluation showed as the

preponderant peak the one attributed to ΔDi-diS_B. As is known, only the intact structure of dermatan sulphate above a certain molecular size is recognised by the enzyme chondroitinase ABC.

As is clear from Table I and Fig. 3, some RO-DS fractions, in the molecular weight range between 6000 and 11000 Daltons, have a surprising activity on HCII. These fractions, as shown by the analysis of the constituent disaccharides, are particularly rich in disulphated disaccharides (Δ Di-diS_B)

d) Obtaining a fraction (I) through controlled acid hydrolysis

2.4 g of a product similar to that obtained in the LL 108/5 fraction of Example 1 are treated with 9 ml of strongly acid cation exchange resin AG50 W-X4 (BioRad) in form H + (having 1.1 meq/ml of exchange) and 135 mg of NaCl in 24 ml of distilled water. The mass is left under agitation at 37°C for 40 hours. It is filtered. The solution is precipitated with 80% strength ethanol by being left to stand for one night at 4°C. The precipitate is collected, redissolved in 4 ml of water and reprecipitated with ethanol at 4°C. The precipitate is collected, redissolved and reprecipitated at r.t. with 75% strength ethanol. The product coded GC 80/4 is obtained. From integration of the signals of the ^{1}H-NMR spectrum (Fig. 4) and the 13C NMR spectrum (Fig. 5) the sequence (X) (with p = 2) of Scheme 1 is obtained.

EXAMPLE 2

200 mg of fraction LL 108/5 of Example 1 are dissolved in 10 ml of 0.1N HCl in a test tube with closed cap, in a waterbath at 60°C. Samples of 2.5 ml

are taken at 0.5, 2, 4 and 6 hours. The solutions taken are neutralized with NaOH at pH 7, then analysed by HPLC for molecular weights. The profiles in Fig. 6 are obtained. The appearance during hydrolysis of discrete molecular weights of 2900, 2300 and 1670 Daltons is clear. This example demonstrates that the hydrolysis time is an important parameter for the purposes of obtaining the oligosaccharides of the invention. With excess hydrolysis time, destruction of the said oligosaccharides clearly occurs.

EXAMPLE 3

13.12 g of RO-DS, obtained as described in Example 1.b, are dissolved in 656 ml of water to which 80 ml of strongly acid resin AG 50 W-X8 in H^+ form (BioRad) at 100-200 mesh are added. The reaction mass agitated in a waterbath at 60°C. 3.75 g of NaCl are also added. The pH falls until it is stabilised at 1.1. The reaction mass is heated for a total of two hours. Lastly the solution is filtered from the resin. The resin is washed with 200 ml of distilled water and the entire solution neutralized at pH 7 with NaOH. The solution is concentrated in a rotary evaporator to 70 ml and an NaCl molarity of about 1M. It is then fractioned by gel-filtration on Ultrogel AcA202 (IBF) in a 5 x 90 cm column, at a flow rate of 2 ml/minute. A 1 M NaCl solution is used as the mobile phase.

20 ml fractions are collected. Void volume is discarded. Eluents are monitored by a spectrophotometer equipped with continuous flow chamber reset to 205 nm. Fractions from 13 to 30 (360 ml, A), from 31 to 44 (280 ml, B) from 45 to 60 (320 ml, C) and from 61 to 67 (140

ml, D) are pooled.

Pool A is concentrated to 55 ml and desalted on Trisacryl GF 05 M (IBF) in 5 x 38 cm column.

The fractions A_1 and A_2 are collected and lyophilized. Pool B is concentrated to 50 ml and desalted on Trisacryl GF 05 M. The fractions B_1, B_2, B_3 are collected and lyophilized.

Pool C, too, is concentrated and desalted. It originates, after lyophilization, the fractions C_1, C_2, C_3.

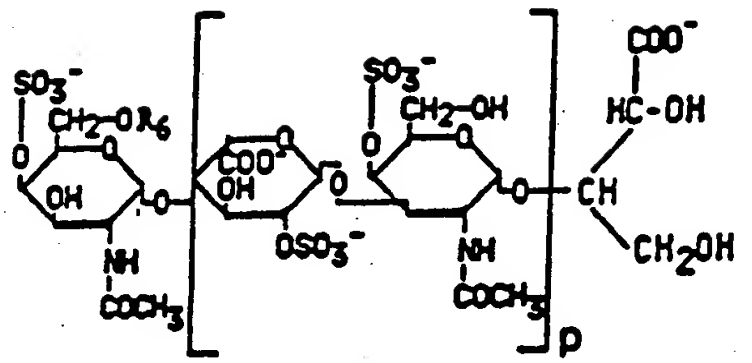
Table II reports the yields, the experimental molecular weights calculated by HPLC on Protein Pak 60-125 columns (Waters), specific rotations and experimental values of SO^-_3/COO^-.

TABLE II

Oligosaccharides obtained by demolition of dermatan sulphate of this invention. Experimental values.

	%Yield	MW	$[\alpha]^{20}_D$	SO^-_3/COO^-
A_1	0.2	3000	nd	nd
A_2	3.4	2900	-22.2°	1.78
B_2	3.0	2300	-26.2°	1.76
B_3	3.3	1500		1.65
C_2	50	550		1.07

Fractions A_2 and B_2 supplied the 13C-NMR spectra shown in Figs. 7 and 8 respectively. The ^1H-NMR spectrum of the A_2 fraction is also given in Fig. 9. The NMR spectra and the other experimental observations agree with the oligosaccharide structures indicated in formula (X), and specified in Table III.



(X)

where p takes the values indicated in Table III.

TABLE III

Theoretical values of oligosaccharide sodium salts

	P	R_6	MW	SO_3^-/COO^-
A_1	4	SO_3^-	2986	2.0
A_2	4	H	2882.9	1.8
B_2	3	H	2277.9	1.75
B_3	2	H	1673	1.67
C_2	0	H	462	1

EXAMPLE 4

Biological activity

The fractions B_2 and B_3, obtained in Example 3, were shown to have low activity on HCII (85.21 ± 9.8 and 32.9 ± 10.5 U/mg respectively), from 3 to 8 times less than the activity of the RO-DS fractions from which they originate.

These fractions on the other hand proved to be very active in the inhibition tests on the cytoplasmatic movements of calcium.

The effect of the oligosaccharides of the present invention on platelet aggregation in a mixed cellular suspension of platelets (10^8/ml) and polymorphonucleate leukocytes (PMN) (5 x 10^6/ml) was evaluated by pre-

incubating the samples with different concentrations of the compounds (1-100 µg/ml). The cellular suspensions were subsequently stimulated with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) (1 µM). As shown in Table IV all the compounds examined led to dose-dependent inhibition of platelet aggregation mediated by PMNs activated by fMLP; semi-maximal inhibition was observed at concentrations ranging from 1 to 10 µg/ml for the oligosaccharide fractions B_2, B_3 and A_2.

The effect of the oligosaccharides of the present invention on the cytoplasmatic movements of platelet Ca^{2+} was evaluated by preincubating the mixed cellular suspensions of platelets and PMNs with different doses of the compounds (1-100 µg/ml). The samples were subsequently stimulated with fMLP (1 µM). As in the case of aggregation, this parameter of platelet activation was also inhibited dose-dependently by all the compounds examined (Table IV). The oligosaccharides B_2, B_3 and A_2 completely inhibited the cytoplasmatic movements of Ca^{2+} in concentrations of about 50 µg/ml, while for the dermatan sulphate of Example 1 and for heparin, taken as reference, complete inhibition was obtained at concentrations higher than 200 µg/ml.

TABLE IV

	% Aggregation vs control	% Calcium vs control
Fraction B_2 µg/ml		
1	58±6	46±6
10	9±1	7±2
50	4±2	4±3
100	9±2	0±0
Fraction B_3 µg/ml		
1	47±5	42±3
10	7±4	9±2
50	3±1	4±3
100	3±2	0±0
Fraction A_2 µg/ml		
1	60±5	48±7
10	12±6	9±3
50	9±2	2±1
100	3±5	0±0
Heparin µg/ml		
1	65±3	88±7
10	54±4	83±6
50	39±2	52±2
100	31±4	37±11
Dermatan sulphate from Example 1 µg/ml		
1	71±4	75±5
10	68±3	63±7
50	55±3	51±4
100	49±4	39±6

EXAMPLE 5

100 kg of pig skin are ground and added to 100 l of 0.5 M NaCl solution. pH is adjusted to 7.0 with sodium hydroxide. 400 g of proteolytic enzyme called Maxatase® are added. Reaction mass is stirred in a reactor for 8 hours at 60°C. It is heated to 80°C and centrifuged in a liquid-liquid separating centrifuge. Fat phase, about 35 kg is discarded. Aqueous phase is filtered through dicalite filter aid. Filtrate is percolated on a chromatographic column (internal diameter: 10 cm, 125 cm high) containing 10 liters of Lewatit MP 50 A (Bayer), a macroporous strong anoinic exchange resin activated in form of Cl^-. Percolate is discarded. The resin is washed with three volumes of 0.65 NaCl solution. The product is eluted from resin with two volumes of 2.0 N NaCl. The obtained solution is concentrated and desalted by inverse osmosis up to 1 molarity of NaCl. Dermatan sulphate is precipitated with two volumes of methanol, collected and dried 67.9 g of product, almost pure, is obtained. The product, further purified, gives the product coded OPD 950-965 having the following characteristics: Sulphur = 5.63%, uronic acids = 32.74%, SO_3/COO^- = 1.04, $[\alpha]^{20}_D$ = - 65.5°, disaccharidic composition obtained by enzymatic attack and separation by HPLC: Δ DiOS = 0.6%, Δ Di6S = 1.0%, Δ Di4S = 90%, Δ Di2,4diS = 7.2%, Δ Di4,6diS = 1.2%. ^{13}C.NMR spectrum, besides the peculiar signals at δ 177.05 ($\underline{CO}$-CH_3), δ 175.79 ($\underline{CO}$-IdoAp6), δ 104.52 (IdoAp1), δ 103.52 (GalpNAc 1), δ 81.69 (IdoAp 4), δ 77.44 (GalpNAc 4), δ 76.87 (GalpNAc 3), δ 76.02 (GalpNAc 5), δ 72.72 (IdoAp 3), δ 70.98 (IdoAp 2, 5), δ

62.48 (GalpNAc 6), δ 54.45 (GalpNAc 2), δ 24.05 (CO-$\underline{CH_3}$), shows the following minor signal: I^*_1 (δ 102), I^*_2 (δ 80), I^*_4 (δ 74), I^*_3 (δ 69.5), I^*_5 (δ 68.5) corresponding to iduronic acid 2-sulphate, and also signals at δ 105.3 (GlucAp 1), δ 75.4 (GlucAp 3), δ 74.0 (GlucAp 2), corresponding to glucuronic acid.

The product OPD 950-965 is oxidized with $NaJO_4$, reduced with $NaBH_4$ as described in Example 1b. The derivative coded RO-DS is obtained. 20 g of this derivative are hydrolyzed in 1000 ml of 0.1 M HCl for two hours as described in example 2. Solution is cooled, neutralized with NaOH and concentrated to 50 ml by rotary evaporator. The solution is desalted on Trisacryl GF 05 M (IBF) in a chromatographic column (internal diameter: 5 cm, 38 cm high). The column effluent is monitored by U.V. detector set at 205 nm. Elution peak is collected, and the tail containing mainly the compounds XI and XII (see Scheme 1) is discarded. The solution is concentrated to 50 ml and oligosaccharides are separated by Exclusion Molecular Chromatography on AcA 202 (IBF) in a column 90 cm high (i.d. diameter 5 cm). 1 M NaCl is used as mobile phase. The operating procedures of Example 3 are followed. The fractions reported in Table V are obtained.

TAB. V. Oligosaccharides from pig skin dermatan sulphate.

Code	Yield* %	P**	MW** d	SO_3^-/COO^-	$[\alpha]^{20}_D$	aHCII potency ****
P4	4.3		1150	1.37	-16.88°	n.d.
P3	1.9	2	1800	1.49	-26.94°	0.03
P2	3.6	3	2200	1.44	-38.78°	0.11
P1	1.1	4	2800	n.d.	n.d.	0.20

Notes

* Yield: calculated on parent DS.

** P: number of disaccharides as indicated in compound X in Scheme I.

*** Molecular weights calculated by HPLC as described in Table I Note.

****HCII potency determined by Stachrom Kit (Stago) in water in comparison with DS of example 1 of patent application no. MI 91 A 002310 filed on 28.8.91the activity of which (162.0+/-9.4 U./mg) was evaluated by calibration curve by using 4th Int.I Standard having 193.4 U./mg.

The fraction P2 in the ^{13}C.NMR spectrum shows all the signals as reported in Figure 8. Besides, it shows signals at δ 73.8 (GluAp 2), δ75.3 (GluAp 3), δ 105.3 (GluAp 1), relevant in the glucuronic acid present in the oligosaccharide fragment in a 30% amount of uronic acids.

100 mg of the fraction P2 are fractionated by S.A.X. HPLC on 10 µ Spherisorb 7.5 x 300 mm column (Sep Phase U.K.) with mobile phase consisting of increasing concentrations of NaCl at a flow rate of 1.5 ml/minute. Collected fractions are desalted and lyophilized. Their characteristics are reported in Table VI.

TABLE VI

Oligosaccharides from pig skin DS, separated by SAX HPLC

Code	% Yield	P	MW	SO_3/ COO^-	HCII potency
113 P2 0M	0.8	3	2100	1.25	n.d.
113 P2 0.5M	O.8	3	n.d.	n.d.	n.d.
113 P2 0.75M	1.05	3	2280	1.75	0.2
113 P2 1.00M	0.94	3	2280	1.75	0.21

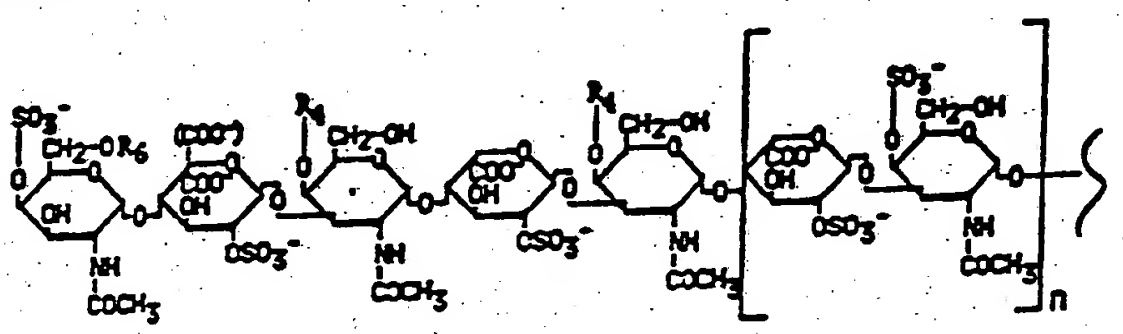
Note

%Yield: calculated on parent Dermatan sulphate

The ^{13}C.MNMR and ^{1}H.NMR spectra of the fraction 113P2/1M confirm its structure of heptasaccharide+Remnant composed solely of Iduronic acid 2-sulphate and N-acetylgalactosamine 4-sulphate.

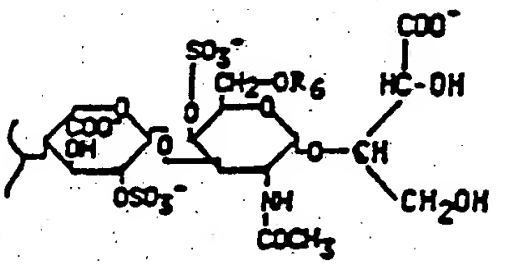
CLAIMS

1. Oligosaccharides of dermatan sulphate having pharmacological activity, characterised by the general formula (I)



Q R S T U V W

(I)



X Y Z

in which:

n = 0, 1 or 2;

R_4 = SO_3^- or H;

R_6 = H or SO_3^-;

Z = remnant of demolition of a uronic acid;

Q, S, U, W and Y = galactosamines;

R, T, V and X = residues of iduronic acid 2-sulphate, at least one of them however being possibly a residue of glucuronic acid 2-sulphate.

2. An oligosaccharide according to Claim 1 , selected from the group consisting of QRSTUXYZ, QRSTUVWXYZ, QRSTUVWVWXYZ, STUVWXYZ, UVQXUZ, SRQXYZ; where Q, R, S, T, U, V, W, X, Y and Z are defined as above.

3. Oligosaccharides according to Claims 1 and 2, characterised by the 13C-NMR spectra in Figs. 5, 7 and 8, an SO_3^-/COO^- ratio between 1.65 and 2.0, and a

molecular weight lying between 3500 and 1000 Daltons.

4. Process for the production of the oligosaccharides according to Claims 1-3, characterised in that a dermatan sulphate with a high content of ΔDi-diS$_B$, is oxidised with sodium periodate, reduced with sodium borohydride and hydrolysed with inorganic acid.

5. Process according to Claim 4, characterised in that the hydrolysis is carried out by a "buffer" system consisting of a polysulphonic resin, in H^+ form, in the presence of a concentration of salt such as to give the optimal hydrolysis pH.

6. Process according to Claims 4 and 5, characterised in that the optimal pH is 1-2 and the optimal reaction time is 0.5-3 hours.

7. Process according to Claims 4-6, characterised in that the oligosaccharide fractions obtained are separated by molecular exclusion chromatography, and, if necessary, by anion exchange chromatography.

8. Pharmaceutical compositions containing as the active principle one or more oligosaccharides of dermatan sulphate according to Claims 1-3.

9. Compositions according to Claim 8, with antiarthritic, anti-inflammatory and platelet antiaggregating activity.

10. Compositions according to Claim 8 for parenteral or oral or topical administration, in the form of injectable sterile solutions or suspensions, compressed capsules, syrups, or creams or ointments.

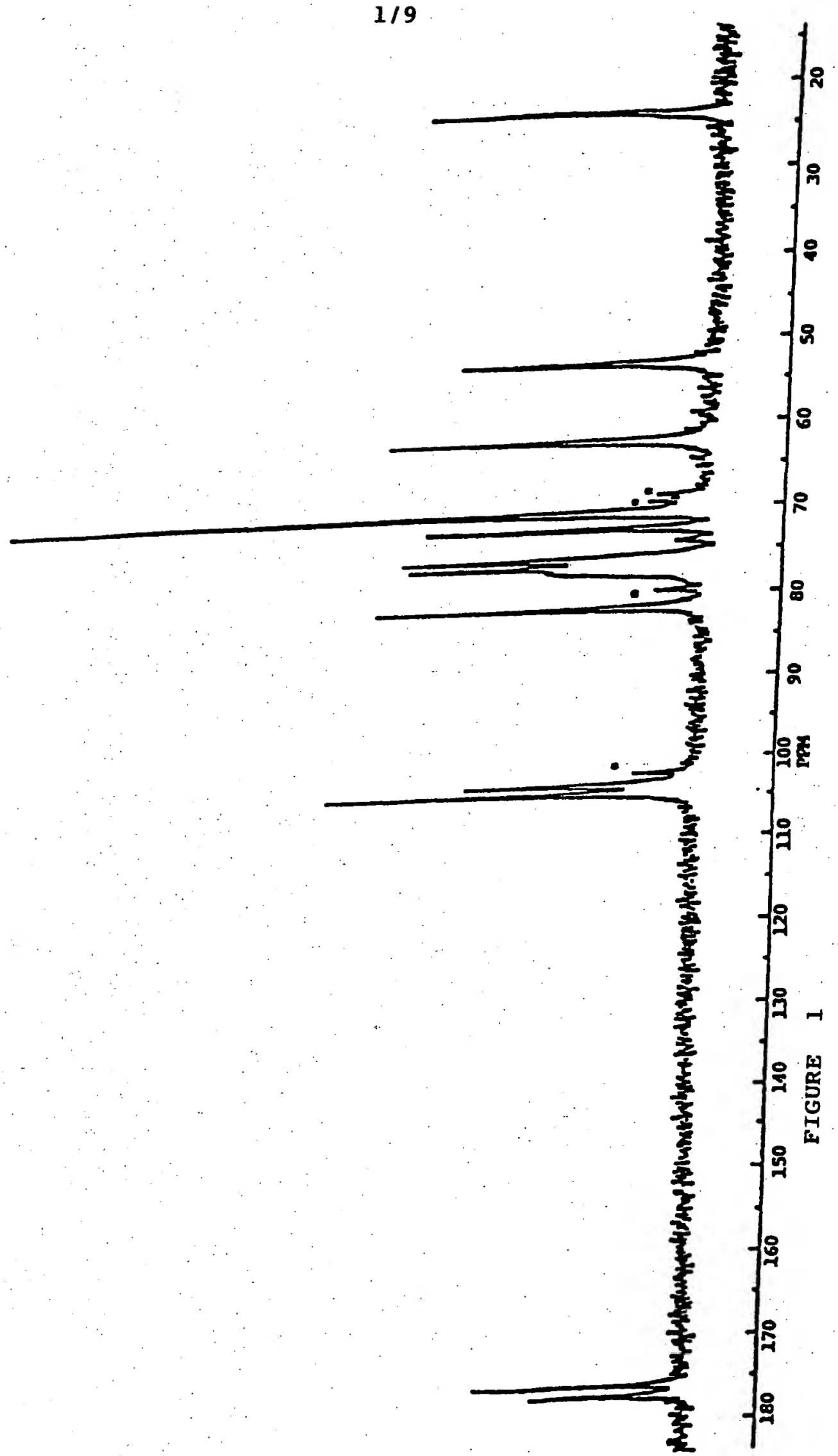


FIGURE 1

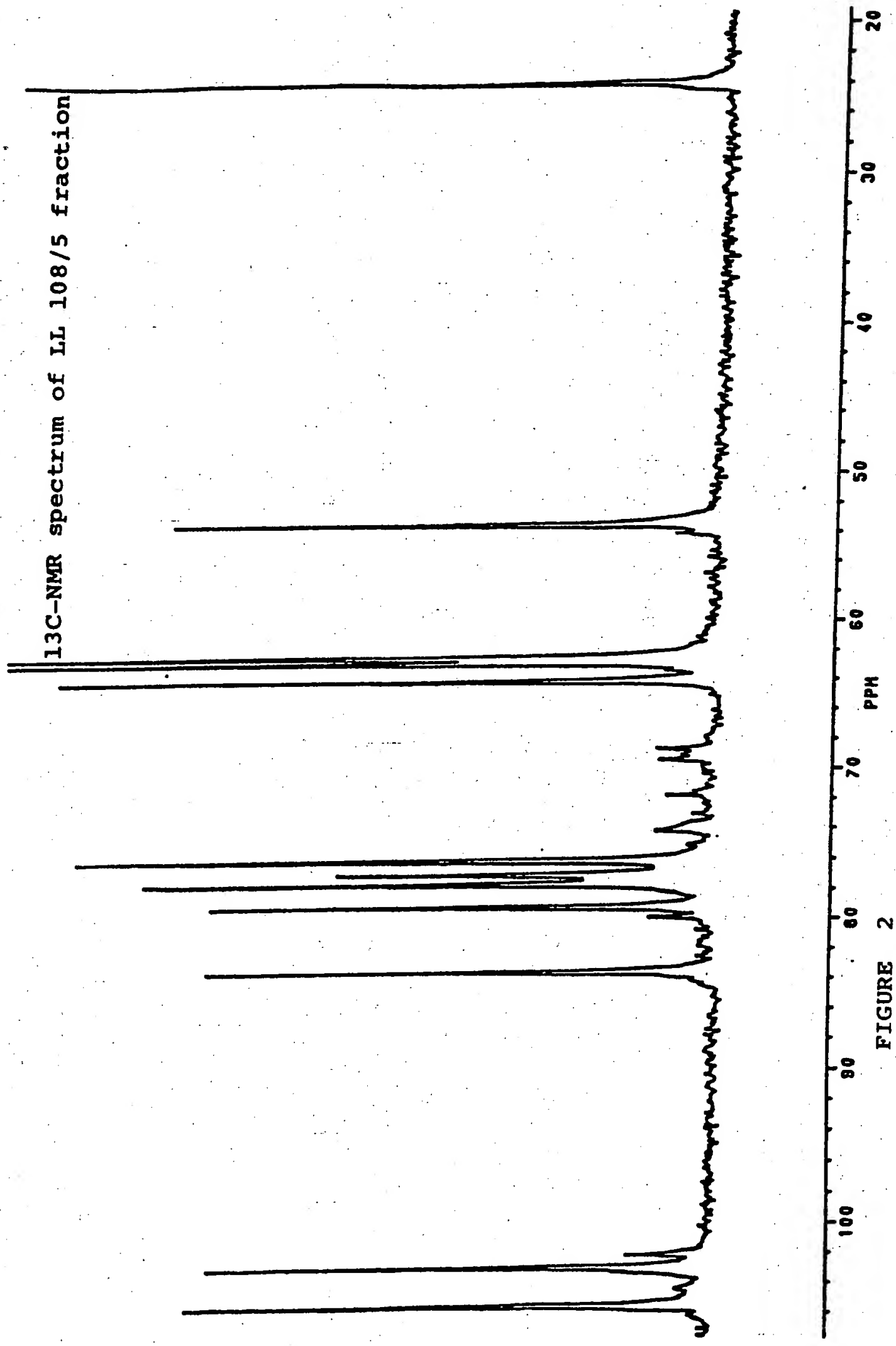

13C-NMR spectrum of LL 108/5 fraction
20
30
40
50
60
70
80
90
100
PPM

FIGURE 2

Comparison between activity of DS and RO-DS on HC II (U mg) as a function of the molecular weight MW in Kd.

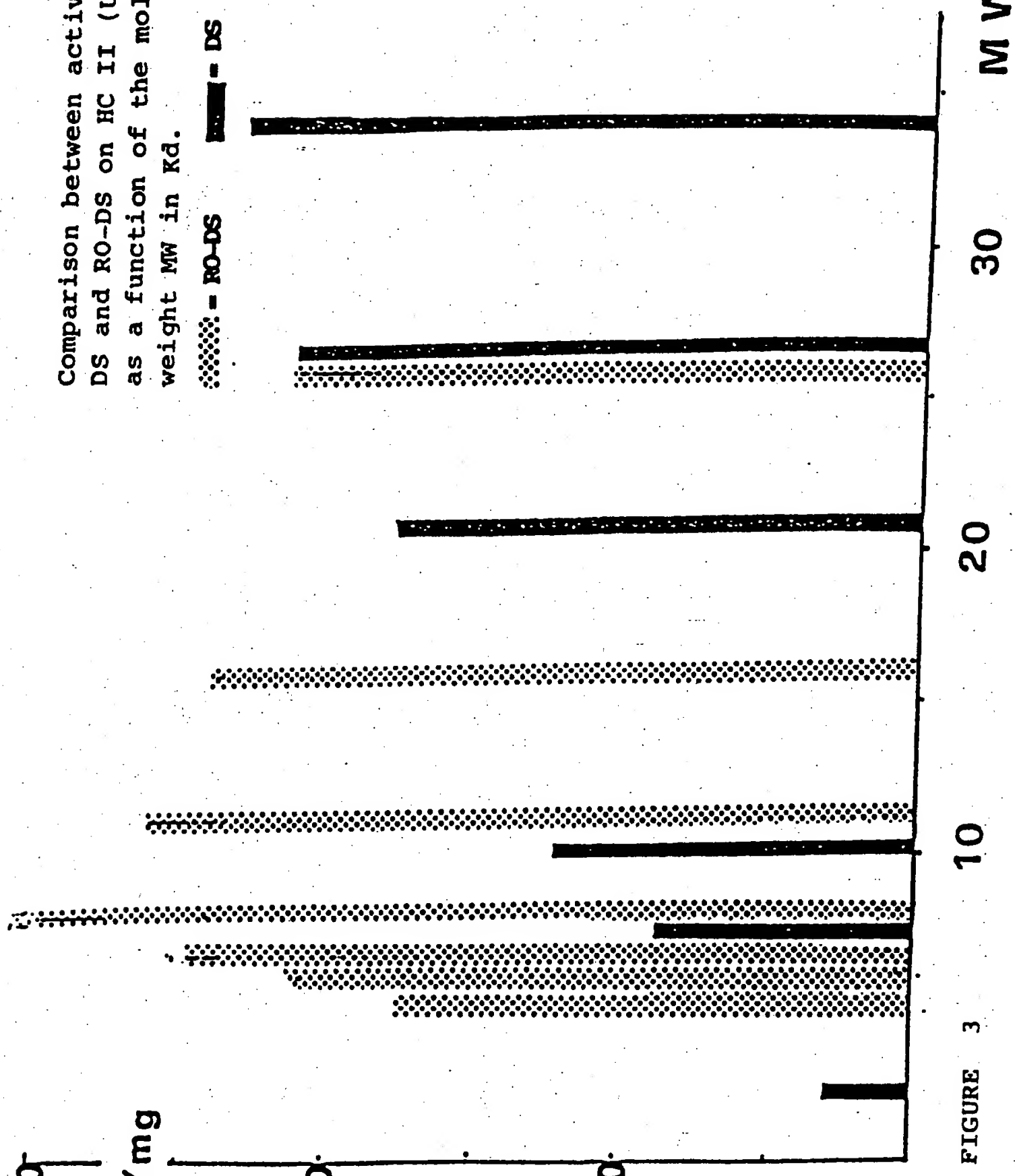


FIGURE 3

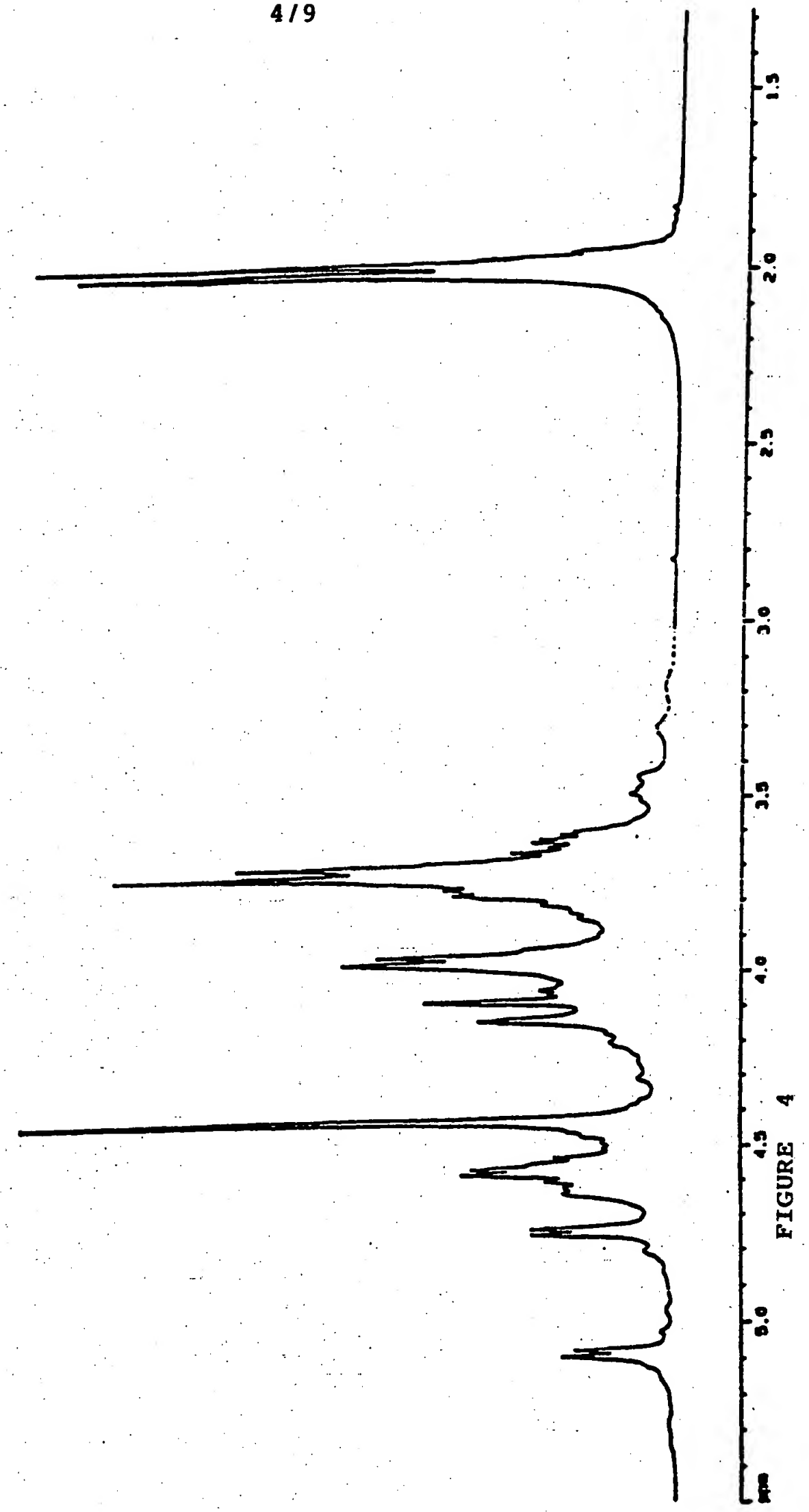

GC 80/4 320K ^{1}H-NMR spectrum of GC80/4 fraction
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
ppm

FIGURE 4

13C - NMR spectrum of GC80/4 fraction

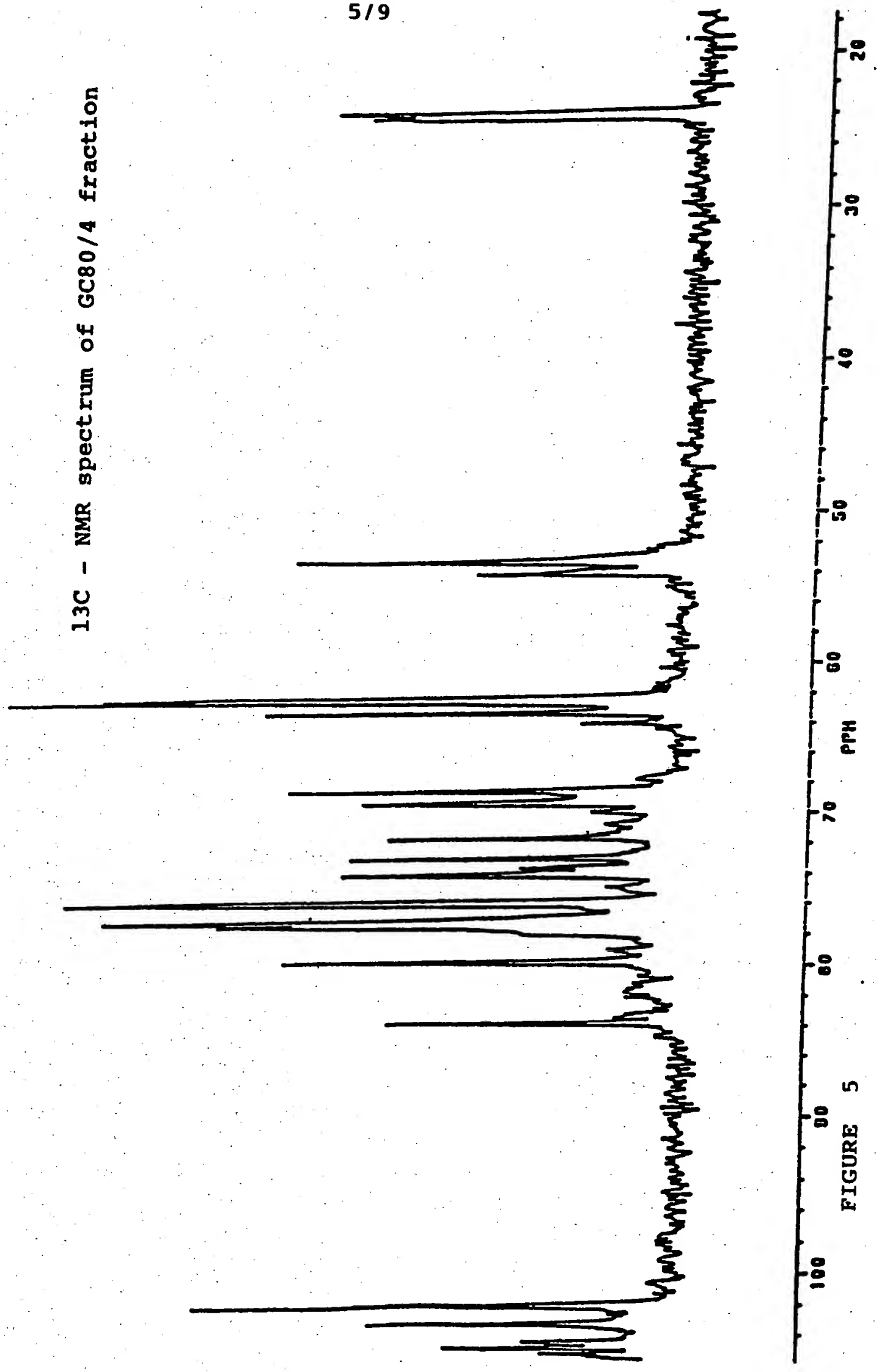


FIGURE 5

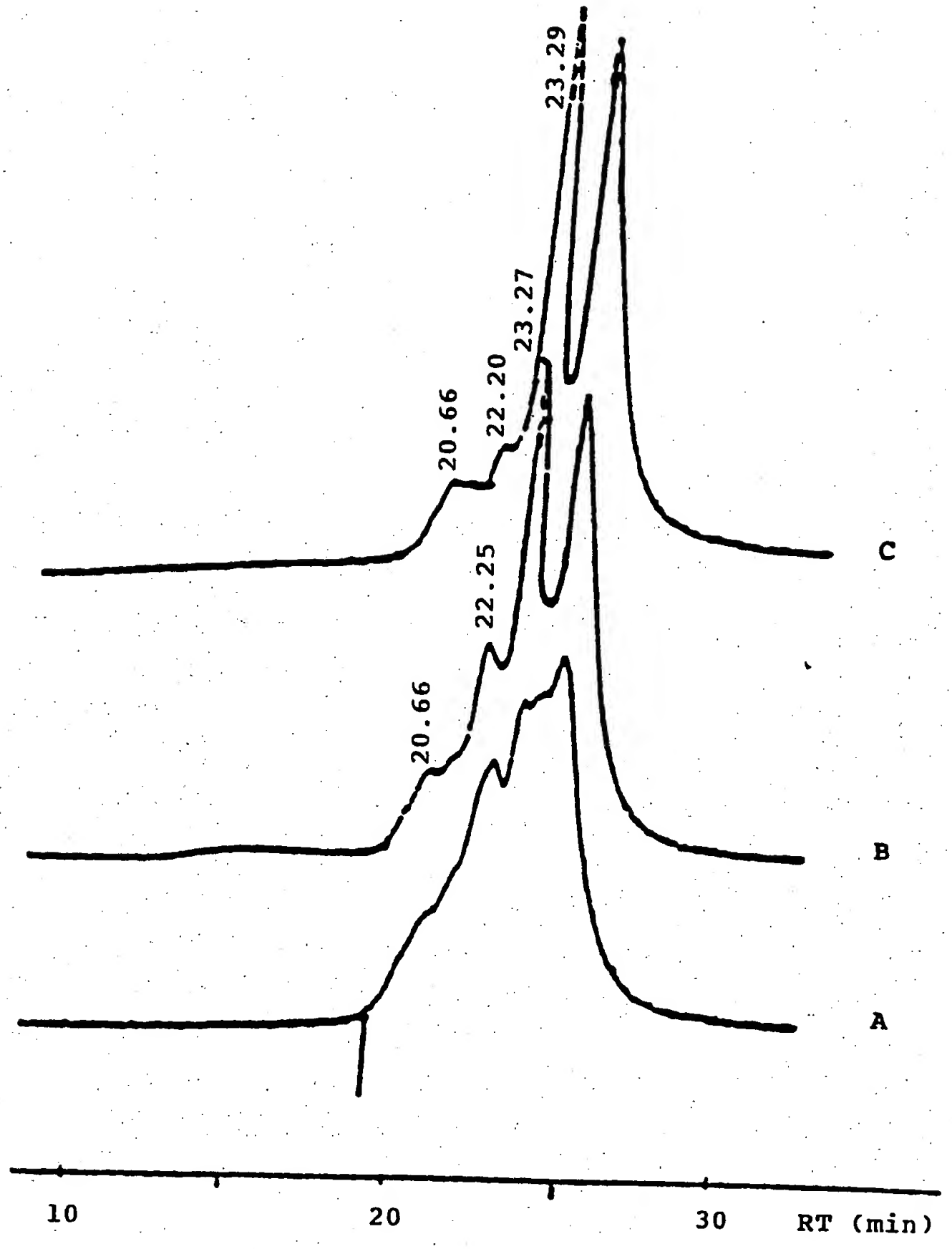


FIGURE 6

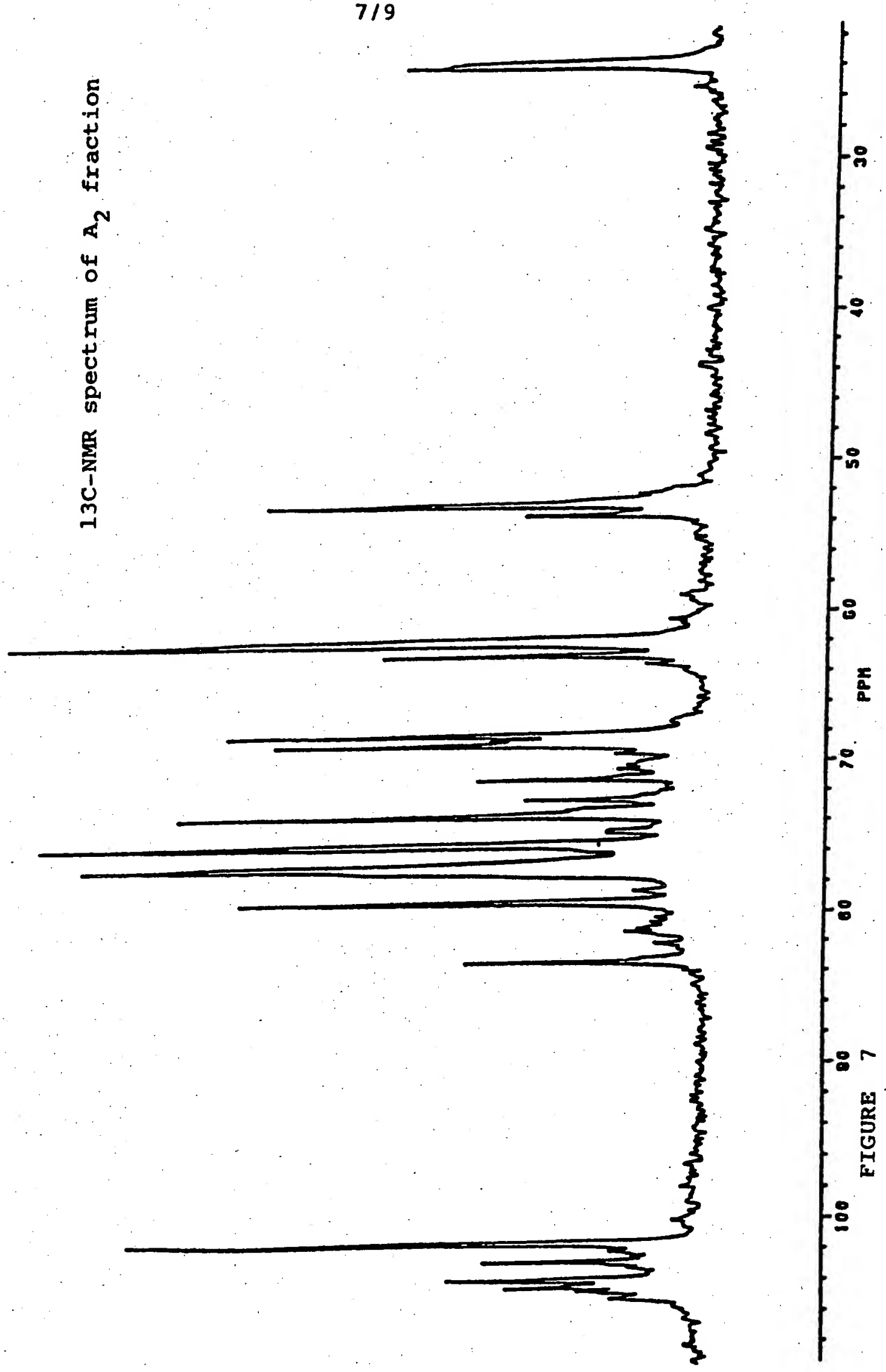


FIGURE 7

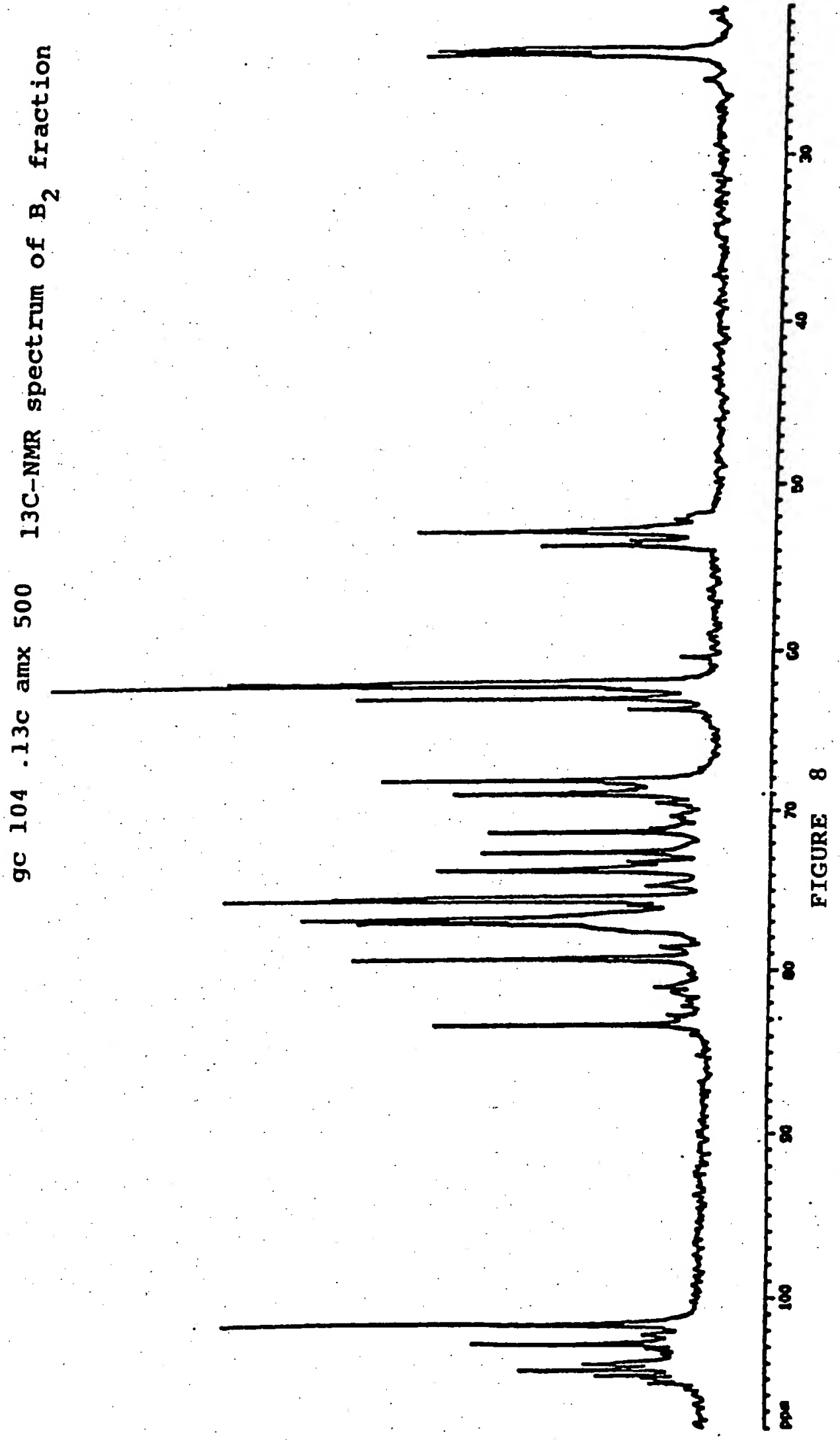

gc 104 .13c amx 500 13C-NMR spectrum of B_2 fraction
30
40
50
60
70
80
90
100
PPM

FIGURE 8

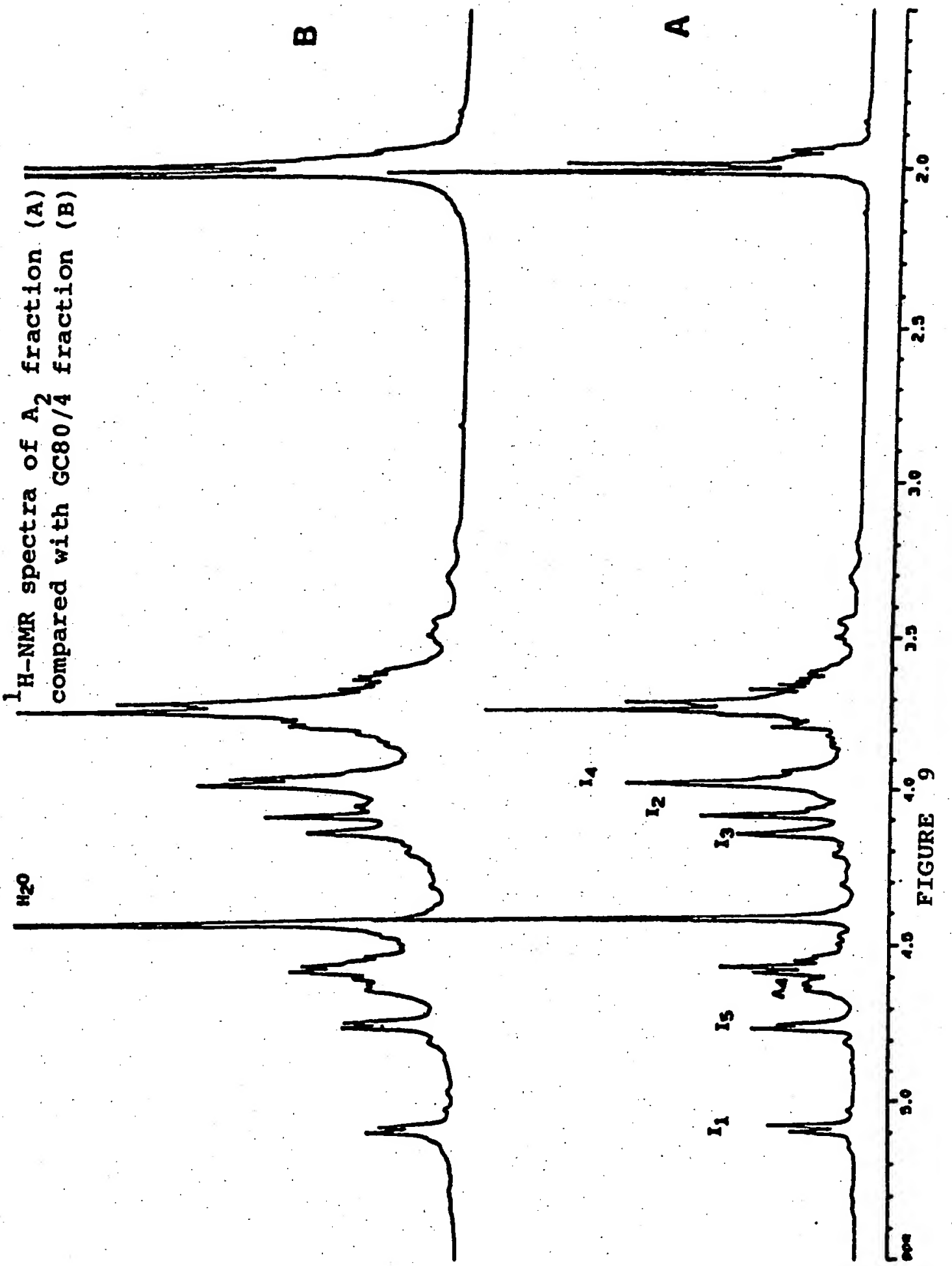

^{1}H-NMR spectra of A_2 fraction (A)
compared with GC80/4 fraction (B)
B
A
H_2O
I_1
I_5
I_4
I_2
I_3
2.0
2.5
3.0
4.0
4.5
5.0

FIGURE 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/01791

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C08B37/00; A61K31/725; A61K31/73

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C08B ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	WO,A,9 004 607 (OPOCRIN SPA) 3 May 1990 cited in the application see abstract see page 1, line 22 - page 4, line 16 see example 1 ---	1,4,8-10
P,A	WO,A,9 115 217 (WASHINGTON UNIVERSITY) 17 October 1991 see page 3, line 18 - page 4, line 11; claims ---	1-7

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 NOVEMBER 1992	19. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MAZET J.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 261, no. 19, 5 July 1986, BALTIMORE, MD US pages 8854 - 8858 D. M. TOLLEFSEN ET AL. 'Molecular size of dermatan sulfate oligosaccharides required to bind and activate heparin cofactor II' see abstract	4-7
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 30, 25 October 1990, BALTIMORE, MD US pages 18263 - 18271 M. M. MAIMONE ET AL. 'Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity' cited in the application see abstract see page 18268, right column, line 22 - line 26	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9201791 SA 63771

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/11/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9004607	03-05-90	AU-A-	4801290	14-05-90
		EP-A-	0439555	07-08-91
		JP-T-	4502928	28-05-92
WO-A-9115217	17-10-91	AU-A-	7742591	30-10-91